

Potential Anti-HIV Activity of *Jatropha curcas* Linn. Leaf Extracts

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Abstract

Objectives: The presence of drug-resistant HIV is a major global concern and warrants the development of novel anti-virals as alternative and inexpensive therapy. In the current study, potentially drug-resistant HIV was isolated and previously unreported anti-viral activity of *Jatropha curcas* Linn. leaf extracts was assessed.

Methods: HIV isolation was done using *in vitro* micro co-culture methods followed by drug susceptibility assays to determine resistance to Zidovudine (AZT), Lamivudine (3TC) and Stavudine (d4T). Soxhlet apparatus was used for extraction of metabolites from leaves of *Jatropha curcas* Linn. and Methanolic and Aqueous Extracts were chosen for further study. Secondary metabolites were detected by High-Performance Thin Layer Chromatography and *in vitro* cytotoxicity established by MTT assay. The extracts were then used in post- and pre-infection studies by measuring inhibition of HIV replication to determine anti-viral activity.

Results: Seven HIV isolates were obtained (isolation rate: 23.33%) with drug IC₅₀ values ranging from 0.001418-82.73 μM AZT, 2.645-15.35 μM 3TC and 18.55-66.23 μM d4T. Tannins, Flavonoids, Saponins were detected in Aqueous Extract and Flavonoids, Saponins in Methanolic Extract. The CC₅₀ values were 32.07 mg/mL and 35.5 mg/mL for Aqueous and Methanolic Extracts respectively. Anti-viral activity was evaluated by inhibition of HIV replication as determined by HIV p24 antigen ELISA. Post-infection (4 isolates) interaction studies showed IC₅₀ values ranging from 0.0255-0.4137 mg/mL and 0.00073-0.1278 mg/mL for Aqueous and Methanolic Extracts respectively and pre-infection (1 isolate) interaction studies showed 100% inhibition by Methanolic and 97.19% inhibition by Aqueous Extract at 25 mg/mL each.

Conclusions: HIV isolates potentially resistant to AZT/3TC/d4T were obtained and *Jatropha curcas* Linn. leaf extracts showed effective anti-viral and probable entry inhibition activity against potentially drug-resistant HIV, which has not been reported earlier. The study indicates that *Jatropha curcas* Linn. is a good candidate for anti-HIV therapy with further research.

Keywords: *Jatropha curcas* Linn., Leaf extract; Anti-HIV activity; Drug-resistant HIV; Secondary metabolites

Introduction

People living with HIV/AIDS often choose traditional or complementary and alternative medicine to complement or replace conventional treatment. The presence of multi-drug or even multi-class resistance in HIV also warrants the need to explore additional means to combat HIV and provide further justifications for the need of alternative and complementary medicines in the treatment of HIV/AIDS.

Most of the traditional systems of medicine in India include some form of 'medicinal plant', herbs or natural plant products. It is therefore not surprising that the activity of these traditional medicines against HIV can be scientifically analysed to deduce the role of natural plant products in their anti-HIV activities. A number of medicinal plants have been reported to have anti-HIV properties [1]. Over the past two decades, substantial progress has been made in research on the natural products possessing anti-HIV activity. A variety of secondary metabolites obtained from natural origin showed moderate to good anti-HIV activity [2].

Jatropha curcas Linn., (colloquially known as errand/danti/katri in Marathi) is a multipurpose, drought resistant, perennial plant that belongs to Euphorbiaceae family, widely distributed in the wild or semi-cultivated areas in Central and South America, Africa, India and South East Asia. The genus name 'Jatropha' derives from the Greek word 'jatr'os' (doctor) and 'troph'e' (food), which implies medicinal uses [3]. Practically all parts of *Jatropha* have some use including green manure, soil erosion control, soap production from oils, leaves

as animal feed, biocidal activity from natural products, leaves and/or stems for sericulture and vermiculture and the more famously known biodiesel production from oil apart from its numerous medicinal uses from various parts [4,5]. All parts of *Jatropha* (seeds, leaves and bark) have also been used in traditional medicine and for veterinary purposes for several centuries [6]. Some of the known medicinal properties of *Jatropha curcas* Linn. include antitumor activities, molluscicidal, insecticidal and fungicidal properties. The seed oil (Jamalgota) can be applied to treat eczema and skin diseases and to soothe rheumatic pain [6]. The latex from branches have been found to be strong inhibitors to watermelon mosaic virus and the leaves and latex are used in healing of wounds, refractory ulcers, and septic gums and as a styptic in cuts and bruises [7]. Topical application of *Jatropha* root powder in paste form is a common ethnobotanical practice for the treatment of inflammation being followed by Bhil tribes from Rajasthan area in India and the methanol extract of these roots exhibited systemic and

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Received October 24, 2013; Accepted November 18, 2013; Published November 20, 2013

Citation: Dahake R, Roy S, Patil D, Rajopadhye S, Chowdhary A, et al. (2013) Potential Anti-HIV Activity of *Jatropha curcas* Linn. Leaf Extracts. J Antivir Antiretrovir 5: 160-165. doi:10.4172/jaa.1000082

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significant anti-inflammatory activity in acute carrageenan-induced paw oedema in albino mice [8]. Four antitumor compounds, including jatropham and jatrophone, are reported from other species of *Jatropha* [8]. The plant has also been homoeopathically used for cold sweats, colic, collapse, cramps, cyanosis, diarrhoea, leg cramps [7,8]. Some other potential antimicrobial activity of *Jatropha curcas* Linn. have also been found [9,10].

Although several studies have shown the antimicrobial activity, there are a very limited number of previous reports on the specific anti-HIV activity of *Jatropha curcas* Linn. One such study showed that *Jatropha curcas* Linn. had specific *in vitro* anti-RT enzyme activity and that the water extract of the branches also strongly inhibited HIV induced cytopathic effects with low cytotoxicity [9]. Several unknown active components of plants belonging to the family Euphorbiaceae (*Jatropha curcas*, *J. multifida*, *Spirostachys africana* and *Trigonostema xyphophylloides*) were found to possess anti-HIV activities by inhibiting HIV-1 cell entry [11-13]. Another study that looked at traditional medicines in the management of HIV/AIDS in Tanzania found that *Jatropha curcas* Linn. leaves were being used for treatment in HIV related conditions such as skin rash and oral candidiasis [14].

With this background, the current study was designed to study drug-resistant HIV-1 in the patient cohort in Mumbai. A small sample size was chosen to evaluate the presence of drug-resistant HIV-1 by phenotypic drug resistance assays. Alternative strategies such extracts from the leaves of medicinal plant *Jatropha curcas* Linn. were evaluated for the potential antiviral and virucidal and/or entry inhibitory activity of these plant extracts.

Materials and Methods

Study population

A total of 30 HIV-seropositive patients (both on Antiretroviral therapy (ART) and drug naïve) attending the ART centre at AIDS Research and Control Centre (ARCON), Sir JJ Hospital Campus, were included in the study. Institutional Ethics Committee approval and written informed consent was acquired prior to blood collection.

HIV isolation

Isolation of HIV from blood samples was done using a PBMC micro-co-culture assay [15]. Briefly, healthy, HIV-seronegative donor PBMCs were stimulated with the mitogen phytohemagglutinin-P (PHA-P; at a final concentration of 5.0 µg/mL; Sigma-Aldrich Corporation, Bengaluru, India), in the presence of human interleukin 2 (IL-2; Sigma-Aldrich) for 24-72 hours before use to promote blast formation and replication of T-cells. The cells were counted and 1×10^6 PHA-stimulated donor cells and 1×10^6 patient PBMCs (samples) were added in duplicate wells of a 24-well tissue culture plate. The final volume was adjusted to 2.0 mL with growth media containing RPMI-1640 (GIBCO, Invitrogen BioServices India Pvt. Ltd, Bengaluru, India) with 20% FBS (Sigma-Aldrich) and 10.0 U/mL IL-2. The plate was then incubated at 37°C with 5% CO₂ in a CO₂ incubator (New Brunswick Scientific, Connecticut, USA). On day 7 and 14, the cultures were replenished with fresh growth media containing 5×10^5 PHA-stimulated donor cells (feeder cells). The culture was continued until day 21 and then terminated. Supernatant fractions from duplicate wells from day 14 and 21 were saved separately, and stored at -70°C until analysis for HIV p24 antigen by ELISA using a HIV-1 p24CA Antigen Capture Assay procured from the AIDS & Cancer Virus Program, National Cancer Institute, Frederick, Maryland, USA as per the manufacturer's instructions. Cultures were considered positive

only when day 21 supernatants showed an increase in p24 antigen concentrations measured in pg/mL from day 14 supernatants.

Drug susceptibility assay using zidovudine (AZT), lamivudine (3TC) and stavudine (d4T)

Drug susceptibility assay was carried out by using a standardized PBMC susceptibility assay [15,16] with slight modifications. Since we were working with small volumes of virus (micro-culture), an additional step of 50% Tissue Culture Infectious Dose (TCID₅₀) calculation was not required. This modification results in a faster and less expensive assay [17]. Dilutions of 2X concentrations (20.0 µM, 2.0 µM, 0.2 µM, 0.02 µM and 0.002 µM) of the drugs of interest (AZT, 3TC and d4T) were prepared by in Growth Medium. The 2X drug working solutions were then added to their respective wells in a 96-well microtitre plate with PBS in the corner wells to help maintain humidity. A single row of uninfected cells and drug was included to serve as a drug toxicity control. Next, 1- to 3-day old PHA-stimulated normal donor PBMC were re-suspended in Growth Medium to a concentration of 4×10^6 PBMC/mL. A volume of 100.0 µL of virus stock was added to 1.0 mL of these donor cells. After mixing gently, the suspension was incubated for 1-3 hours at 37°C, 5% CO₂ with humidity to allow the virus to infect cells. After incubation, the cells were washed to remove any uninfected HIV and 100.0 µL of infected cells was dispensed into the well of the microtitre plate containing the appropriate 2X drug concentrations. The final volume in each well was 200.0 µL. The plates were then incubated at 37°C, 5% CO₂ with humidity. On day 7, 20.0 µL of the supernatant from each of the drug susceptibility assay wells was added to the lysing solution (10% Triton X-100™; Sigma-Aldrich) and used for determining the p24 antigen levels. The 50% Inhibitory Concentration (IC₅₀) values were calculated by the median effect equation using GraphPad Prism software (GraphPad Software, Inc., California, USA). The potential phenotypic drug resistance was determined by calculating the fold increase in IC₅₀ values of the drugs against the HIV isolates compared with IC₅₀ values against a standard strain of HIV (HIV-1_{IIIIB}).

Collection, identification and authentication of *Jatropha curcas* Linn.

The plant material was collected from Rajaramnagar (Islampur), Sangli District, Maharashtra. Since the plant material was collected from wild, it needed to be authenticated prior to further use. The identification and authentication of the plant was done at Blatter Herbarium, Department of Botany, St. Xavier's College, Mumbai and a voucher specimen was deposited in the herbarium (Specimen no. K.V.S. 2298).

Solvent extraction of plant material and detection of secondary metabolites

Extraction of leaves from *Jatropha curcas* Linn. was carried out using a Soxhlet apparatus and various solvents in increasing order of polarity. The solvents used for extraction were hexane, dichloromethane (DCM), methanol (Sigma-Aldrich) and water (Distilled). Briefly, *Jatropha curcas* Linn. leaves were thoroughly washed with water, shade dried on a clean filter paper and ground into fine powder. A total of 30.0 g of leaf powder was wrapped in a thimble made of Whatman filter paper no. 1, and was placed into the main extraction unit of the Soxhlet apparatus. A round bottom flask containing solvent was placed on a heating mantle and temperature adjusted to the boiling point of each solvent. Once the solvent in the side arm became colourless, the

extraction process was stopped. The desired extract containing small amount of solvent was taken in a clean and dry pre-weighed petriplate and the solvent was evaporation completely. The extracts were then stored in a cool and dry conditions until further *in vitro* assays.

Detection of the secondary metabolites of the plant extracts was carried out by High Performance Thin Layer Chromatography (HPTLC) on a third-party commercial basis (M/s Anchrom Test Lab (I) Pvt. Ltd., Mumbai) using a Linomat 5 Semi-automatic Sampler and densitometric evaluation done using Camag TLC Scanner and Visualizer. The documentation was done using winCATS Planar Chromatography Manager software.

Cytotoxicity assay

Before using the *Jatropha curcas* Linn. extracts for antiviral assay it was necessary to assess their *in vitro* toxicity against human cells. The cytotoxicity of experimental moieties (ranging from 200 mg/mL to 6.25 mg/mL) was determined using both Vero cell lines as well as PBMCs by MTT assay [18]. The 50% cytotoxic concentration (CC₅₀) values were then calculated using GraphPad Prism software.

Assays to assess potential antiviral activity

Potential antiviral and entry inhibitory activity of the experimental moieties was assessed using standardized drug susceptibility assays using PBMCs and measured by inhibition of HIV p24 antigen. Two types of assays were carried out: Post-infection interaction (cell-associated virus) to determine antiviral activity and a preliminary Pre-infection interaction (cell-free virus) to assess probable inactivation of virus (virucidal activity) or entry inhibitory activity.

Post-infection interaction (cell-associated HIV)

The HIV isolate/s were first allowed to infect PBMCs and the plant extracts (ten-fold dilutions ranging from 25.0 mg/mL to 0.025 mg/mL, based on the CC₅₀ values of the extracts) were added to the suspension afterwards. Briefly, a volume of 100.0 µL of viral isolates were individually added to 1.0 mL of PHA-stimulated PBMCs in a 1.5 mL microcentrifuge tube and incubated for 1 hr in a CO₂ incubator to allow the virus to infect the cells. After incubation, the cells were washed carefully to remove any uninfected HIV and 100.0 µL of infected cells were plated into wells of 96-well tissue culture plate. Next, 100.0 µL of 2X concentrations of different plant extracts were added. The plates were then incubated for 7 days at 37°C in the CO₂ incubator. The contents of each well was transferred to microfuge tubes, cells were pelleted and the supernatants used for determining the p24 antigen levels. The results obtained were then analysed to calculate IC₅₀ values and compared with the IC₅₀ values of standard drugs AZT, 3TC and d4T against the respective isolates.

Pre-infection interaction (cell-free HIV)

The HIV isolate/s were first interacted with the plant extracts (25 mg/mL) for 1 h and then allowed to infect PBMCs. Briefly, a volume of 10.0 µL of viral isolates were individually added to 10.0 µL of 2X concentrations of the plant extracts in a 1.5 mL microcentrifuge tube and incubated for 1 hr at 37°C in a CO₂ incubator. After 1 h of interaction the experimental moiety-virus suspension was added to 96-well tissue culture plate containing 100.0 µL of PHA stimulated PBMCs and incubated for 1 h in the CO₂ incubator to allow the virus to infect the cells. After incubation, the cells were washed carefully to remove any uninfected HIV and the plate was incubated for 7 days in the CO₂ incubator. The contents of each well was transferred to microfuge tubes, cells were pelleted and the supernatants used for determining the

p24 antigen levels. The results obtained were then analysed to calculate p24 antigen inhibition.

Results

HIV isolation by PBMC co-cultivation and determination of phenotypic drug resistance

Thirty samples were processed for Peripheral Blood Mononuclear Cells (PBMC) co-cultivation from which seven HIV isolates were obtained giving an isolation rate of 23.33%. The phenotypic drug resistance was determined by calculating the fold increase in IC₅₀ values of the drugs against the HIV isolates compared with reported IC₅₀ values against a standard strain of HIV (HIV-1IIIB) [19]. The fold increases in IC₅₀ values are given in Table 1.

Solvent extraction and detection of secondary metabolites

Solvent extraction using Soxhlet apparatus yielded in 2.2 g of Hexane extract, 1.5 g of Dichloromethane (DCM) extract, 1.3 g of Methanolic extract and 1.8 g of Aqueous extract from leaves of *Jatropha curcas* Linn. Since the Hexane and DCM extracts were extremely sticky and insoluble in water or RPMI-1640 medium, only Methanolic (ME) and Aqueous extracts (AE) were used for all the further assays.

Secondary metabolites of *Jatropha curcas* Linn. leaf extracts were detected by HPTLC analysis. The plant extracts showed the presence of Tannins, Flavonoids and Saponins in the AE and Flavonoids and Saponins in the ME (Table 2). Figure 1 shows the HPTLC fingerprint of the plant extracts.

In vitro cytotoxicity of plant extracts

The *in vitro* cytotoxicity was carried out using MTT assay and CC₅₀ calculated. The CC₅₀ values of *Jatropha curcas* Linn. plant extracts were 35.49 mg/mL for Methanolic and 32.07 mg/mL for Aqueous extract as shown in Figure 2.

Anti-HIV activity of *Jatropha curcas* Linn. methanolic and aqueous extracts

Anti-HIV activity of Methanolic and Aqueous extracts of *Jatropha curcas* Linn. was studied using the *in vitro* drug susceptibility assay with an endpoint determination of p24 antigen. Percent inhibition of p24

HIV isolate	AZT (µM)	Fold increase	3TC (µM)	Fold increase	D4T (µM)	Fold increase
HI/4C	0.001418	0.01	15.35	64.58	ND	ND
HI/15C	0.03146	0.19	2.645	11.13	66.23	76.18
HI/23C	72.13	445.25	8.041	33.83	20.82	23.95
HI/35C	3.071	18.96	3.503	14.74	45.66	52.52
HI/53C	48.81	301.30	14.41	60.62	33.41	38.43
HI/60C	82.73	510.68	5.403	22.73	18.55	21.34
Standard IC ₅₀	0.162		0.2377		0.8694	

Figures of fold increase in bold type depict resistance.

Table 1: Fold increases in IC₅₀ values of AZT, 3TC and d4T against HIV isolates.

2 ^o Metabolite	Methanolic Extract	Aqueous Extract
Alkaloids	-	-
Tannins	-	+
Flavonoids	+	+
Saponins	+	+

Key: '+' : Detected; '-' : Not detected.

Table 2: Secondary metabolites in methanolic and aqueous extracts of *Jatropha curcas* Linn. leaves.

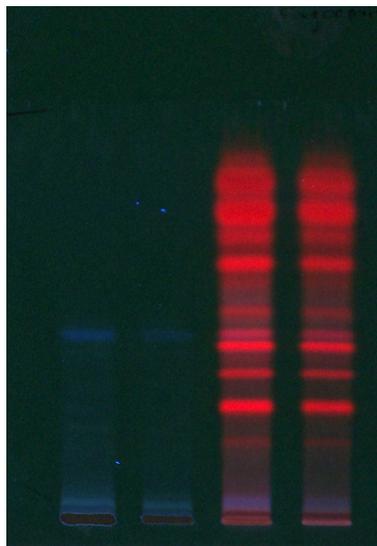


Figure 1: HPTLC Fingerprint of *Jatropha curcas* Linn. extracts at 366 nm wavelength using a Linomat 5 Semi-automatic Sampler and densitometric evaluation done using Camag TLC Scanner and Visualizer. Lane 1 and 2 show the fingerprint for Aqueous Extract while Lane 3 and 4 show the fingerprint for Methanolic Extract.

Calculation of CC₅₀ values of *Jatropha curcas* Linn. Extracts

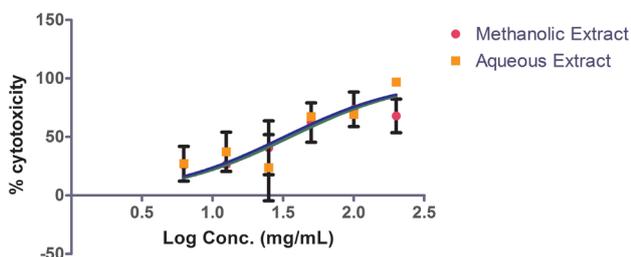


Figure 2: Cytotoxic activity of *Jatropha curcas* Linn. Methanolic (●) and aqueous (■) leaf extracts using MTT assay on Peripheral Blood Mononuclear Cells (PBMCs). The CC₅₀ calculation was done using the GraphPad Prism software.

antigen was determined against 4 isolates, and it was observed that the IC₅₀ values of the Methanolic leaf extracts ranged from 0.00073-0.1278 mg/mL (Figure 3; Selective index = 19095.34 ± 17709.64) while those of the Aqueous leaf extracts ranged from 0.0255-0.4137 mg/mL (Figure 4; Selective index = 705.86 ± 438.33) as shown in Table 3. Since this was a preliminary study, the pre-exposure interaction to assess the potential entry inhibitory activity was carried out only against 1 isolate (15C), and percent p24 antigen inhibition was determined. It was seen that the methanolic extract showed 100.0% inhibition while the aqueous extract showed 97.19% inhibition.

Discussion

The need for novel antivirals for managing HIV/AIDS is indisputable. The nature of the virus to evolve faster than the antivirals being made available has made it imperative that newer approaches to combating the virus are considered.

Our laboratory has been actively involved in the research of novel experimental moieties for their potential anti-HIV activity. Previous

studies carried out in our laboratory with Mangrove plants such as *Ocimum sanctum*, *Withania somnifera*, *Tinospora cordifolia*, *Avicennia officinalis*, *Rhizophora mucronata* and 'Shilajit', a herbomineral [20] and other medicinal herbs such as *Phyllanthus amarus* [21] have shown tremendous potential in their anti-HIV activities. The leaf extracts of medicinal plant *Jatropha curcas* Linn. have not been studied extensively in the past. A preliminary study carried out by our laboratory for assessing the anti-HIV activity of *Jatropha curcas* Linn. extracts showed promising results [22]; hence, in this study an attempt was made to determine the potential anti-HIV activity using potentially drug-resistant clinical isolates of HIV-1.

HIV Isolate	Methanolic extract			Aqueous extract		
	IC ₅₀ (mg/mL)	CC ₅₀ (mg/mL)	Selectivity Index (CC ₅₀ /IC ₅₀)	IC ₅₀ (mg/mL)	CC ₅₀ (mg/mL)	Selectivity Index (CC ₅₀ /IC ₅₀)
HI/15C	0.0007333	35.5	48411.29	0.08375	32.07	382.92
HI/23C	0.1288		275.62	0.4077		78.66
HI/35C	0.02204		1610.70	0.02551		1257.15
HI/53C	0.001361		26083.76	0.02903		1104.71

Table 3: Selectivity indices of *Jatropha curcas* Linn. Extracts.

IC₅₀ value calculation of *Jatropha curcas* Linn. methanolic extract

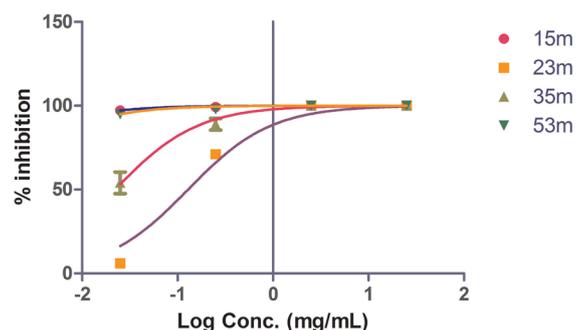


Figure 3: Inhibitory activity of *Jatropha curcas* Linn. methanolic leaf extracts against 4 HIV isolates: 15 (●), 23 (■), 35 (▲) and 53 (▼) in the post-infection interaction study. The suffix 'm' after the isolate number indicates 'methanolic extract'. The IC₅₀ calculation was done using the GraphPad Prism software.

IC₅₀ value calculation of *Jatropha curcas* Linn. aqueous extract

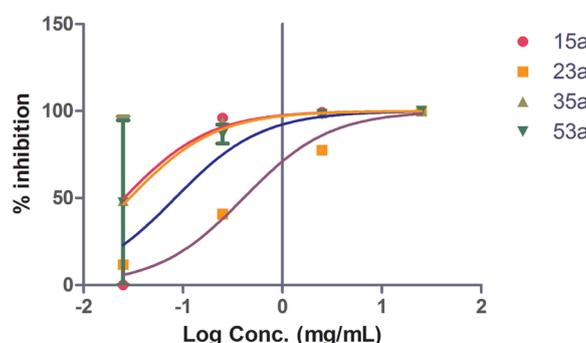
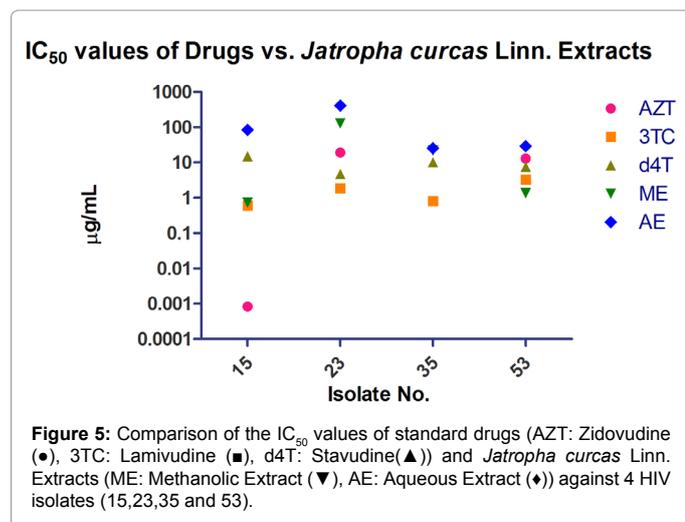


Figure 4: Inhibitory activity of *Jatropha curcas* Linn. aqueous leaf extracts against 4 HIV isolates: 15 (●), 23 (■), 35 (▲) and 53 (▼) in the post-infection interaction study. The suffix 'a' after the isolate number indicates 'aqueous extract'. The IC₅₀ calculation was done using the GraphPad Prism software.



The plant extracts showed effective antiviral activity as compared to standard drug AZT, 3TC and d4T (Figure 5) in HIV p24 antigen inhibition assays. The aqueous leaf extracts showed IC₅₀ values ranging from 0.0255-0.4137 mg/mL and the methanolic leaf extracts showed IC₅₀ values ranging from 0.00073-0.1278 mg/mL; while in the pre-infection studies, the aqueous leaf extracts showed 97.19% p24 inhibition and the methanolic leaf extract showed 100.0% p24 inhibition at 25 mg/mL each indicating potential denaturation of virus (virucidal activity) or entry inhibitory activity.

These findings are quite significant since the extracts seem to work in both pre- and post-infection regimes and hence suggest that the effect may be mediated by a direct action on the virus particle. Perhaps it blocks binding or uptake to cells or causes immediate particle lysis. While we have planned experiments to elucidate a preliminary mode of action to evaluate the future potential of the extracts, it would be unreasonable to expect a precise mechanism at this stage.

Previous studies using *Jatropha curcas* Linn. stem bark extracts have shown inhibition of HIV-induced cytopathic effects [9,23]. Recently, the anti-HIV effect of 12-deoxyphorbol-13-phenylacetate, a compound synthesised from *Jatropha* phorbol esters, has been demonstrated; it inhibits HIV entry into target cells [24,25]. The anti-HIV activity of *Jatropha curcas* Linn. may be attributed to the secondary metabolites present in the leaf extracts. The aqueous leaf extract showed the presence of Tannins, Flavonoids, and Saponins as detected by HPTLC, while Flavonoids, Saponins were detected in the methanolic leaf extract. Recent reports by Kamal et al. [26] have given the chemical composition of leaves as containing Cyclic triterpenes: stigmaterol, stigmast-5-en-3, 7 diol, stigmast-5-en-3,7 diol, cholest-5-en-3,7 diol, campesterol, sitosterol, 7-keto-sitosterol as well as the d-glucoside of sitosterol and Flavonoids: apigenin, vitexin, isovitexin. Leaves also contain the dimer of a triterpene alcohol (C₆₃H₁₁₇O₉) and two flavonoidal glycosides. Further fractionation and purification of these secondary metabolites for active antiviral and entry inhibitory activity screening is required to determine the exact mode of action.

While our laboratory has also shown similar potential anti-influenza (inhibition of hemagglutinin protein of Influenza virus) activity of the *Jatropha curcas* Linn. plant extracts [27], to the best of our knowledge, this is the primary report of anti-HIV activity of *Jatropha curcas* Linn. methanolic and aqueous leaf extracts against drug-resistant HIV.

The study also showed that the *Jatropha curcas* Linn. methanolic and aqueous leaf extracts were equally effective against isolates that were sensitive and potentially resistant to standard anti-retroviral drugs AZT, 3TC and d4T.

Conclusion

To conclude, the study has evaluated that *Jatropha curcas* Linn. has prospective antiviral activity against potentially drug-resistant HIV-1 and that these experimental moieties have favourable implications on the prevention or management of HIV/AIDS. The plant extracts may be used in the formulation of microbicides or surface disinfectants. It can be therefore deduced from that study that *Jatropha curcas* Linn. is a good candidate for anti-HIV therapy with further research.

Acknowledgements

The authors would like to thank the staff of ARCON, Mumbai for their assistance in collection of blood samples.

Author Disclosure Statement

The authors declare that there is no conflict of interests regarding the publication of this article.

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